

The use of feline herpesvirus and baculovirus as vaccine vectors for the *gag* and *env* genes of feline leukaemia virus

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The *env* and *gag* genes from feline leukaemia virus were expressed in a thymidine kinase-negative feline herpesvirus and a baculovirus. Cats were vaccinated with various combinations of these recombinant viruses and

100% protection against feline leukaemia virus challenge was achieved using an immunization schedule which utilized both *env* and *gag* products delivered at both a mucosal and systemic site.

Introduction

Feline leukaemia virus (FeLV) was isolated from a lymphoma cluster which occurred in a group of cats in Scotland (Jarrett *et al.*, 1964). The virus has since been shown to be represented by three subgroups which are characterized by the seroreactivity of their external glycoproteins (Sarma & Log, 1973). They cause not only lymphomas, leukaemias and myeloproliferative disease, but also immunodeficiency syndromes and aplastic anaemias (Hardy, 1980). Early attempts to produce a vaccine for FeLV infection included the administration of live or inactivated FL74 feline lymphoma cells (Theilen *et al.*, 1969) using a variety of adjuvants. Many of these attempts were unsuccessful in protecting cats either against persistent viraemia or secondary lymphoma and sarcoma development. Soluble tumour cell antigen vaccines are presently commercially available and other experimental vaccines based on immune-stimulating complexes and subunits have also been reported (Osterhaus *et al.*, 1989). This report assesses the use of both a live virus vector, feline herpesvirus (FHV), and a subunit expression system (baculovirus), as a means of vaccinating cats with both the *env* and *gag* proteins of FeLV.

Methods

Animals. Specific pathogen-free cats were used in all experiments. Animals were housed in a containment facility either in individual cages or in pens of six to eight cats. Animals received cat food and water on an *ad libitum* basis.

Expression of FeLV *gag*, *gp85* and *gp70* in FHV. The expression of FeLV *gag* and *gp85* in FHV has been described previously (Cole *et al.*, 1990). For expression of the *gp70* portion of the *gp85 env* gene, an oligonucleotide was designed having three in-frame termination codons. This oligonucleotide was ligated into the *gp85* coding sequence at the *AluI* site at nucleotide 7298 (Donahue *et al.*, 1988) and the coding sequence for the p15E protein was eliminated, resulting in the production of a truncated *gp70* protein. For insertion into FHV, this *gp70* gene was inserted between the human cytomegalovirus immediate early promoter and the BGH poly(A) signal into the FHVΔtk vector pCG113 (provided by J. Nunberg, Cetus Corporation) to produce the plasmid p113-70B. This was cotransfected into Crandell feline kidney (CRFK) cells with DNA from FHV UT88. Recombinant viruses were isolated by standard techniques (Nunberg *et al.*, 1989). Expression of the *gp70* protein was confirmed by Western blotting and immunoprecipitation of [³⁵S]methionine-labelled proteins from infected cells using monoclonal antibodies (MAbs) to *gp70* (data not shown).

Expression of FeLV *gag*, *gp85* and *gp70* in baculovirus. For expression of the *gag* protein in *Autographa californica* nuclear polyhedrosis virus (AcNPV), the method described in the accompanying paper (Thomsen *et al.*, 1992) was used. Recombinant baculoviruses were selected and expression of the *gag*, *gp85* and *gp70* proteins confirmed by immunoprecipitation of [³⁵S]methionine-labelled proteins from infected cells using MAbs specific to *gag* or *gp70* (data not shown).

Preparation of antigen for use as a vaccine. *Spodoptera frugiperda* (Sf9) cells in spinner flasks were infected at a cell density of 1×10^6 cells/ml at a multiplicity 5 p.f.u./cell. The cells were infected with the following recombinant viruses: either AcNPV *gp85* or AcNPV *gp70*, or coinfecting with AcNPV *gp85* and AcNPV *gag*. Each culture was harvested at 72 h post-infection (p.i.). Cells infected with *gp85* or *gp70* were tested as vaccines. Infected cells were separated from the culture medium by low-speed centrifugation and stored frozen until used. *Gag/gp85* particles were purified from 100 ml of the supernatant of infected cells by centrifugation at 100 000 *g* for 1 h. The pellet from this procedure was resuspended in 5 ml PBS and stored frozen prior to formulation as a vaccine.

FHV recombinants expressing either *gp70*, *gp85* or *gag* were grown in CRFK cells and harvested 3 days p.i. After clarification to remove cell debris, virus in the supernatant fluid was titrated and stored at -70 °C before use.

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Laboratory assays. P27 levels in cats were measured using a commercially available kit produced by Synbiotics. P27, a group-specific antigen of FeLV, is found in the blood of persistently infected animals and hence can be used as a test for viraemia. Standardization in our laboratory indicated that within the context of known positive and negative sera, absorbance values of ≥ 0.25 are positive for viraemia.

FeLV-specific antibodies were measured in two ELISAs against purified gp70 and purified whole virus. Gp70 was prepared by affinity chromatography using disrupted virus from FL74 cells (Theilen *et al.*, 1969) and a MAb (a gift from Dr N. C. Pedersen, Davis, Ca., U.S.A.). Column elutes were concentrated and used to coat ELISA plates at 2 µg protein/ml. After incubation for 18 h at 4 °C, plates were washed in PBS before the addition of cat sera diluted 10-fold in PBS containing 0.05% Tween 20 (PBST). An appropriate dilution of horseradish peroxidase-labelled anti-cat immunoglobulin (Kirkegaard and Perry) was then reacted at room temperature with the plate for 45 min. After a further wash step, substrate and chromogen were added and colour was allowed to develop before reading of the plates on an ELISA reader. Each plate contained appropriate negative and positive controls. Sera were tested in duplicate and increases in absorbance values of at least twice pre-treatment levels were scored as positive. Virus for the whole virus assay was prepared from FL74 cells by filtration through an Amicon filter and gradient centrifugation (Akerblom *et al.*, 1989). Material was stored at -20 °C and titrated with known positive and negative sera to find the appropriate dilution for use. Assays were run in a fashion similar to that described for gp70 antibody detection.

Vaccine experiment 1. Two groups of cats were used in this experiment. The first group ($n=6$) was inoculated twice intranasally/orally (day 0 and 21) with 1.0 ml of FHVΔtkgp85 containing 1.3×10^5 p.f.u. and 10^7 p.f.u. respectively. (This was accomplished by using a syringe to which a small nasal cannula had been attached. Animals were gently restrained and approximately 0.25 ml was dropped into each nostril and 0.5 ml placed towards the back of the oropharynx.) The second group ($n=6$) was inoculated intramuscularly on day 0 with

8×10^6 AcNPVgp85-infected Sf9 cells and at 21 and 33 days with 10^7 AcNPVgp85-infected Sf9 cells. Animals were challenged intranasally/orally with 1 ml of supernatant fluid from confluent CT600 cells (a gift from Dr N. C. Pedersen). This was administered on days 0, 2, 4 and 7. On day 5, cats were immunosuppressed with Depo-Medrol (Upjohn) used at 5 mg/kg body weight. Day 0 of challenge was 40 days after the initial vaccination. Six unvaccinated cats were challenged at the same time to act as controls.

Vaccine experiment 2. A single group of animals ($n=17$) was used in this experiment and they were inoculated intranasally/orally with 1.0 ml of FHVΔtkgp70 containing 2×10^5 p.f.u. Twenty-seven days later, all animals were revaccinated with the same preparation. Thirty-nine days after the second vaccination, cats received 2.5×10^7 Sf9 cells infected with AcNPVgp70 via intramuscular injection. Sixty-four days after this inoculation, cats were divided into two subgroups. The first subgroup ($n=10$) was immunosuppressed and challenged as described for experiment 1. The second subgroup ($n=7$) was placed in a pen with eight persistently FeLV-infected cats and six unvaccinated negative controls.

Vaccine experiment 3. Seven groups of animals were used in this experiment. In the first group ($n=8$), cats received two intramuscular inoculations of Sf9 cells infected with baculovirus AcNPVgp70 at a 29 day interval. In the second group ($n=8$), animals received two inoculations of Sf9 cells. The first consisted of AcNPVgp70 and gp85/gag particles, and the second consisted of gp85/gag particles alone. In group 3 ($n=8$), cats were first inoculated intranasally/orally with FHVΔtkgp85 containing 2×10^5 p.f.u. in a 1 ml dose and 29 days later were intramuscularly inoculated with Sf9 cells infected with AcNPVgp85. Cats in the fourth group ($n=8$) were first coinoculated intranasally/orally with FHVΔtkgp85 and FHVΔtkgag. Each inoculum contained 2×10^5 p.f.u. Twenty-nine days later, animals received an intramuscular inoculation of purified gag/gp85 particles. In group 5 ($n=6$), cats received two intranasal inoculations of 2×10^5 p.f.u. FHVΔtkgp70, 29 days apart. In the last group ($n=8$), animals were

Table 1. Numbers of cats which scored positive in an ELISA using purified FeLVgp70

Experiment	Vaccination/challenge treatment	Number	Number of positive animals*	
			Post-vaccination	Post-challenge
1	FHVΔtkgp85 i.n./inoculation†	6	0	0
	AcNPVgp85 i.m./inoculation†	6	1	0
	None/inoculation	6	0	0
2	FHVΔtkgp70 i.n. AcNPVgp70 i.m./inoculation†	10	3	3
	FHVΔtkgp70 i.n. AcNPVgp70 i.m./contact	7	4	7
	None/inoculation	4	0	0
	None/contact	6	0	6
	AcNPVgp70 i.n./inoculation	8	0	0
3	FHVΔtkgp70 i.n./inoculation	6	0	2
	FHVΔtkgp70 i.n., AcNPVgp70 i.m./inoculation	8	4	5
	FHVΔtkgp85 i.n., AcNPVgp85 i.m./inoculation	8	3	4
	AcNPVgp70/gp85/gag i.m./inoculation	8	2	4
	FHVΔgp85/gag i.n. AcNPVgp85/gag/inoculation	8	2	3
	None/inoculation	8	0	0
	None/inoculation	8	0	0
	None/inoculation	8	0	0

* Animals scores as positive if absorbance values after vaccination or challenge were greater than twice pre-vaccination levels.

† i.n., Intranasally; i.m., intramuscularly.

first inoculated intranasally/orally with a 1 ml dose of FHVΔtkgp70 containing 2×10^5 p.f.u. Twenty-nine days later, animals were intramuscularly inoculated with AcNPVgp70. Forty-four days after the last vaccination, all cats from each group were immunosuppressed/challenged as described in experiment 1. Eight matched, non-vaccinated animals were similarly challenged to act as controls.

Results

In experiment 1 (where cats were inoculated with either FHVΔtkgp85 or AcNPVgp85), none of the animals showed any detrimental response to vaccination. After challenge, animals were bled and p27 levels were assayed as a measure of viraemia. Fig. 1 shows the results from bleeds taken before challenge and at 3, 5 and 7 weeks post-challenge. Of the six control cats (Fig. 1a), five became persistently viraemic and one never became infected. In both of the FHVΔtkgp85 (Fig. 1b) and AcNPVgp85 (Fig. 1c) vaccine groups, four of six animals became viraemic. The levels of viraemia in animals that became infected were not different from controls.

Results of the ELISAs showed that none of the cats vaccinated with FHVΔtkgp85 responded to gp70 or whole FeLV whereas four of six cats vaccinated with AcNPVgp85 responded on the whole FeLV ELISA and one of six responded to purified gp70 (Table 1).

Experiment 2 was designed to test firstly whether exclusion of the p15E subunit would improve efficacy, secondly whether combined mucosal and parenteral priming could enhance the vaccines' protective effect and thirdly whether a natural challenge system would increase the chances of detecting immunity.

The combined vaccine regime caused no detrimental signs in the animals and a larger number of cats, seven out of 17 (Table 1) showed a response to gp70 compared to the previous response to the recombinant viruses given separately. A greater number, 16 of 17, also responded in the whole virus assay (data not shown). Injection challenge established infection in three of four controls but only three of 10 vaccinated animals were p27-positive at 7 weeks (Fig. 2a and b). Contact challenge established infection in only two of six controls whereas only one of six vaccinees showed a very low p27 level at 7 weeks post-challenge.

These results indicate that combined mucosal/parenteral inoculation of gp70 can provide protection against challenge even in the injection/immunosuppression challenge system. The low infection rate of the controls in the contact/challenge system on the other hand make results from the vaccine/contact challenge group difficult to interpret. Experiment 3 was designed to probe further the role of the dual vaccination schedule and to test the importance of the gag protein in immunity. Hence, a

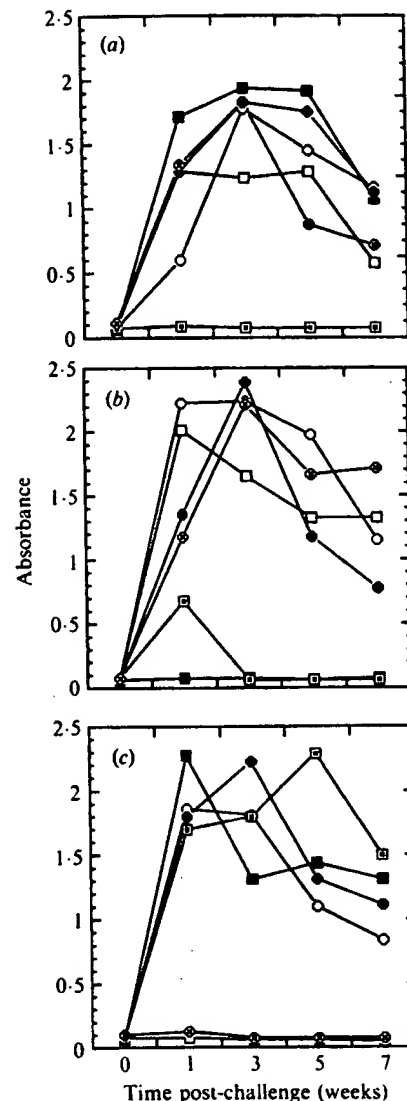


Fig. 1. Assays of p27 in sera taken from cats at 0, 1, 3, 5 and 7 weeks post-challenge (experiment 1). (a) Controls, $n = 6$; (b) FHVΔtkgp85, $n = 6$; (c) AcNPVgp85, $n = 6$.

series of animals were vaccinated with various regimes and, because of the poor infection rates established in controls using contact challenge and the apparent success of the vaccine in the face of immunosuppression, all cats in the last experiment were challenged using the infection/immunosuppression model.

The p27 values for the six vaccine regimes at 9 weeks post-challenge are shown in Fig. 3. Animals treated with gp70 in either vector alone showed some low levels of protection similar to that seen with gp85 in experiment 1. Dual inoculation for mucosal and parenteral immunity with gp70 gave 50% protection, and cats in this group, which were still viraemic at 9 weeks post-challenge, appeared to have lower p27 levels. Dual inoculation of

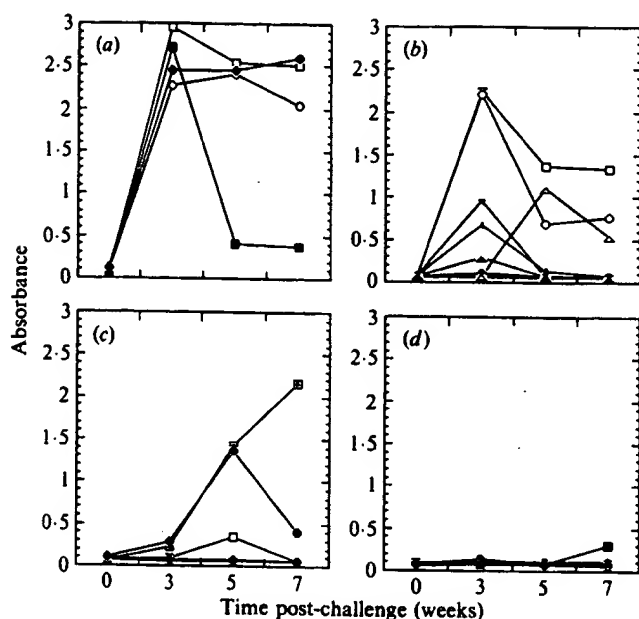


Fig. 2. Assays of p27 in sera taken from vaccinated and challenged cats (experiment 2). (a and b) Injection challenge; (c and d) contact challenge. (a) Control, $n = 4$; (b) FHV Δ tkgp70 plus AcNPV gp70, $n = 10$; (c) control, $n = 6$; (d) same as (b) with $n = 7$.

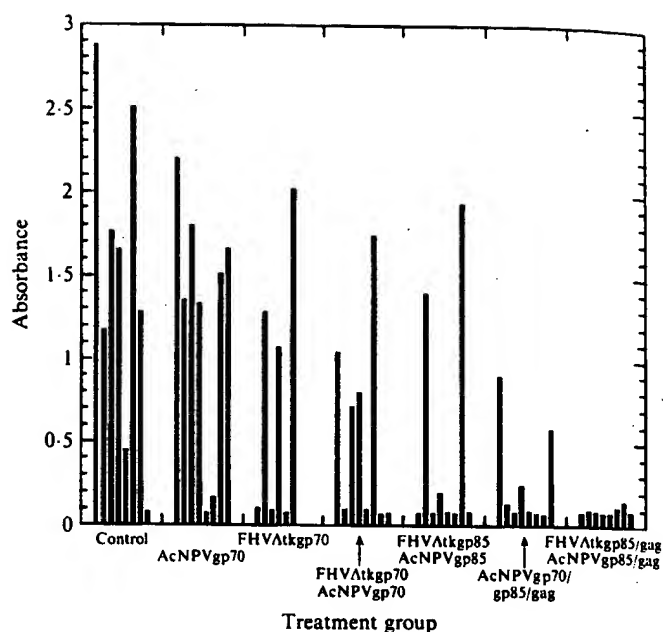


Fig. 3. Results of p27 assays at 9 weeks post-challenge from sera of cats vaccinated with different vector combinations. Each bar represents value from one cat (experiment 3). Values in the shaded portion are considered negative.

Table 2. Serological response from experiment 3 in cats vaccinated with various FHV and AcNPV recombinant viruses and challenged by direct inoculation with supernatant fluid from CT600 cells

Vaccination treatment	Number	Group's mean absorbance in whole FeLV ELISA		
		Pre-vaccination	Post-vaccination (number of positive cats)	Post-challenge
AcNPVgp70 i.m.	8	0.57 \pm 0.026	2.18 \pm 0.49 (8)	2.42 \pm 0.39
FHV Δ tkgp70 i.n.	6	0.35 \pm 0.10	0.43 \pm 0.29 (2)	2.19 \pm 0.85
FHV Δ tkgp70 i.n. AcNPVgp70 i.m.	8	0.36 \pm 0.10	1.68 \pm 0.82 (8)	2.45 \pm 0.59
FHV Δ tkgp85 i.n.	8	0.38 \pm 0.15	1.13 \pm 0.49 (8)	2.25 \pm 0.54
AcNPVgp85 i.m.	8	0.52 \pm 0.16	2.21 \pm 0.35 (8)	2.46 \pm 0.39
AcNPVgp70/gp85/gag i.m.	8	0.35 \pm 0.13	1.83 \pm 0.34 (8)	2.74 \pm 0.23
FHV Δ tkgp85/gag i.n.	8	0.59 \pm 0.27	0.64 \pm 0.28 (0)	1.82 \pm 0.57
AcNPVgp85/gag i.m.	8			
No vaccination	8			
Inoculation challenge				

gp85 improved protection levels and again lowered p27 levels compared to the use of gp70. This indicates that the previously enhanced protection seen with dual site inoculation of gp70, compared to single site inoculation of gp85, is due to immunological stimulation at the two sites rather than the possible immunosuppressive effect of p15E. The inclusion of the gag protein as part of gag/gp85 particles again improved vaccine efficacy despite the fact that only a parenteral application was

used. When both mucosal and parenteral sites were vaccinated using both gp85 and gag then 100% protection was achieved at 9 weeks post-challenge. In this group of eight cats, three animals showed a transient viraemia of between 2 and 4 weeks duration, whereas five animals never became viraemic.

Antibody scores for these groups are shown in Tables 1 and 2. Again, only low numbers appeared positive using the gp70 assay whereas all animals (except cats vacci-

nated with FHV Δ tkgp70 alone) showed post-vaccination seroconversion using the whole virus assay. Levels of response do not appear to correlate with protection; however in groups where some animals were protected and others became viraemic, post-vaccinal titres showed a wide range of values (data not shown) in both of these groups.

Discussion

The most elegant strategies for vaccination against any viral disease would take full account of the pathogenesis surrounding the infection and the antigenic targets which the virus presents to its host. With respect to FeLV, the pathogenesis of the disease is known in great detail and includes horizontally transmitted virus (Hardy *et al.*, 1973), which first replicates in the oropharyngeal lymphoid tissue, and is then disseminated throughout the body via a cell-associated viraemia, and secondarily replicates in systemic lymphoid tissue and bone marrow (Rojko *et al.*, 1979). This leads to a marrow-origin, cell-free viraemia and further tertiary replication in mucosal sites from where it is shed in high concentrations (Francis *et al.*, 1977). Antigenic targets for the virus are less well defined although sites involved in complement-mediated lysis, antibody-dependent cellular cytotoxicity of virus-infected cells (McCarty & Grant, 1983; Grant *et al.*, 1980*a*) and neutralization (Osterhaus *et al.*, 1989; deNoronha *et al.*, 1983; Grant *et al.*, 1980*b*) have been mapped to the gp70 and p15E envelope proteins (Grant *et al.*, 1983; Nunberg *et al.*, 1984; Nick *et al.*, 1990). Studies with analogous viruses would also suggest that both the core gag and env protein can act as important immunological targets for cytotoxic T cells (Tc) (Holt *et al.*, 1986; van der Hoorn *et al.*, 1985; Flyer *et al.*, 1983; Manjunath *et al.*, 1986; Martin & Rouse, 1990). This cytotoxicity would presumably be mediated through an endogenously processed antigen (Germain, 1986) which would arise most effectively through viral replication. This knowledge suggests that vaccines that can engender immunity at any of these sites by utilizing responses to the env and gag proteins of FeLV may be successful in denying FeLV persistence. By constructing vectors which express both these proteins and by delivering them to both mucosal and systemic sites, this work clearly shows that, in the natural host of FeLV, full protection against persistent viraemia can be achieved.

Many different approaches to FeLV vaccination have been reported including complete inactivated virus (Olsen *et al.*, 1977; Schaller *et al.*, 1977), inactivated FeLV-infected cells (Jarrett *et al.*, 1975; Grant *et al.*, 1980*a*), crude and defined subunit preparations (Lewis *et al.*, 1981; Salerno *et al.*, 1978) and immune-

stimulating complexes (Osterhaus *et al.*, 1985). This is the first report of protection from disease when FeLV antigens have been expressed in a vector system and demonstrates the potential usefulness of alphaherpesviruses as vectors. Previous reports with other alphaherpesviruses have shown that a sequence from foot-and-mouth disease virus protein VP1 can be expressed in bovine herpesvirus type 1 (Kit *et al.*, 1991) and that one of the envelope glycoproteins from hog cholera virus expressed in pseudorabies virus can protect pigs against that infection (van Zijl *et al.*, 1991). FHV is particularly relevant in this situation because of the vector's extensive replication in the oropharynx (Gaskell & Povey, 1979). The thymidine kinase-negative (Δ tk) mutant used as the vector did not cause any clinical signs when given to cats despite the virulence of the parent strain (data not shown) and should address the induction of an immune response at the mucosal entry site of the virus. The precise way in which that was achieved was not determined although the differences between the effectiveness of the various immunization protocols (Fig. 3) signify the importance of those responses. There are no reports in the literature of mucosal IgA measurements against FeLV, and although with Sendai virus IgA alone has been shown to afford protection (Manzanec *et al.*, 1987), other systems have failed to show a correlation between IgA levels after vaccination and protection (Kimman *et al.*, 1989). Mucosal stimulation, however, is likely to produce memory cells, which lodge both at the site of priming and at distant systemic sites (Pierce & Cray, 1981, 1982; Lycke *et al.*, 1987). Memory cells at the latter sites would then respond to a systemic boost and hence increase systemic immunity. Local memory cells would also respond on challenge, and as vaccination and challenge occur at the same site in the dually vaccinated cats, such a response might be expected to be important. In other systems where local and systemic vaccination have been compared (Kimman *et al.*, 1989), both have primed for a local response, although with local vaccination memory responses after challenge appear stronger with oronasal vaccination than with systemic vaccination.

In the system described here, local vaccination was still not sufficient to prevent FeLV replication in the majority of cats given FHV expressing gp70 or gp85. The secondary and tertiary rounds of FeLV replication are dominant events in terms of the life history of the natural infection, and hence it might be expected that strong levels of systemic immunity are needed to ensure protection. This is illustrated by the group of cats that received only the systemically administered AcNPVgp85/gag. Protection with this preparation was not as good as that of the dually vaccinated cats (FHV Δ tkgp85/gag and AcNPVgp85/gag; Fig. 3), as two cats remained

viraemic after challenge. However, this group appeared to be very resistant to infection, for as well as showing a high number of animals that failed to become viraemic, the two positive animals showed lower levels of viraemia compared to animals in other groups (Fig. 3). This is particularly noticeable when comparing cats given gp70/gag to those given gp70 alone (Fig. 3), and illustrates the specific usefulness of gag as an immunogen. Whole virus vaccine preparations will obviously contain gag and there are reports of gag-specific antibody in FeLV-recovered animals (Lutz *et al.*, 1980). The immune response(s) that this protein induces is not known although gag proteins from other retroviruses act as a target for Tc (Holt *et al.*, 1986; van der Hoorn *et al.*, 1985; Flyer *et al.*, 1983; Manjunath *et al.*, 1986). Its inclusion in any FeLV vaccine will thus broaden the immune response and if this is through the induction of Tc then it is possible that this response could be more effective against antigenically heterogeneous viruses because of the likelihood that the T cell response will be cross-reactive (Zweerink *et al.*, 1977; Rosenthal & Zinkernagel, 1980; Jeggo & Wardley, 1982). Tc responses are efficiently stimulated through endogenous processing of antigen and hence the differences in protection rates seen between the groups given both live and dead vectors compared to those given only dead vectors could also be explained by a lack of Tc induction.

The quantity and quality of antibody against the env protein are also likely to be important as sub-neutralizing levels of antibody to a number of viruses have been shown to enhance viral infectivity (Halstead, 1988; Takeda *et al.*, 1988). It is possible that this occurs through a number of different mechanisms including antibody-antigen complexes binding to cellular Fc receptors, viral aggregation, or enhancing conformational changes at the receptor binding domain because of antibody binding at neighbouring epitopes. An example of pre-existing antibodies increasing the severity of FeLV has been reported where vaccinated animals showed an increased susceptibility to infection on challenge (Pedersen *et al.*, 1986). In the ELISAs reported here, mean post-vaccinal titres from experiment 3 were highest in the groups of cats given AcNPVgp70 alone or AcNPVgp70/gp85/gag (Table 2). Titres in the group that showed 100% protection were next highest although after challenge their mean titres surpassed the two AcNPV vector groups. Responses to gp70 alone are more difficult to interpret (Table 1) as no clear pattern is apparent. In both cases, however, there appears to be no correlation between antibody titres, expressed either as group means (Tables 1 and 2) or as individual values (data not shown), and protection. In a previous report (Lutz *et al.*, 1980) virus neutralization correlated closely with ELISA values, and neutralization is reported to correlate to

protection (Russell & Jarrett, 1978). Such an association is not clear from this study.

Responses in the whole virus ELISA could presumably also contain a response to the p15E moiety of gp85. The role which this protein plays in the pathogenesis of FeLV infection is still controversial as claims for a potent immunosuppressive effect and hence exclusion from any vaccine preparation lie side by side with arguments for its inclusion in any vaccine because anti-p15E antibodies could have a beneficial effect on protection (Olsen *et al.*, 1977; Mathes *et al.*, 1978, 1979). Antibody reactivity which maps to the N-terminal end of p15E is capable of viral neutralization (Nick *et al.*, 1990) and thus may play a role during the systemic phase of the infection. When we compared FHVΔtkgp85 plus AcNPVgp85 with FHVΔtkgp70 plus AcNPVgp70, the regime which contained p15E gave better protection indicating that in this particular format no deleterious effect of p15E could be detected.

The data from this work show that a vaccine, designed to take into account both the pathogenesis of FeLV and the potentially important immunological responses which the natural host to this disease is likely to make, can provide 100% protection against an agent which is often thought to be particularly intractable to prophylaxis. These strategies are important for the protection of cats against FeLV; present experimental and licenced vaccines do not have this high protection rate.

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(Received 3 January 1992; Accepted 16 March 1992)